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Research article

Epigenetic history of an Arabidopsis trans-silencer locus and a test for relay of trans-silencing activity

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Abstract

Background: Meiotically heritable epimutations affecting transgene expression are not well understood, even and in particular in the plant model species, Arabidopsis thaliana. The Arabidopsis trans-silencer locus, C73, which encodes a fusion protein between the repressor of photomorphogenesis, COP1, and green fluorescent protein (*GFP-COP1*), heritably modifies the expression pattern and *cop1*-like cosuppression phenotypes of multiple *GFP-COP1* target loci by transcriptional gene silencing.

Results: Here we describe three additional features of trans-silencing by the C73 locus. First, the silencing phenotype of C73 and of similar complex loci was acquired epigenetically over the course of no more than two plant generations via a stage resembling posttranscriptional silencing. Second, imprints imposed by the C73 locus were maintained heritably for at least five generations in the absence of the silencer with only sporadic spontaneous reversion. Third, the pairing of two other *GFP-COP1* transgene loci, L91 and E82, showed an increased tendency for epigenetic modification when L91 carried an epigenetic imprint from C73, but not when E82 bore the imprint.

Conclusions: The latter data suggest a transfer of trans-silencing activity from one transgene locus, C73, to another, namely L91. These results extend our operational understanding of interactions among transgenes in Arabidopsis.

Background

Certain genetic loci are known to modify the expression of other allelic or non-allelic partner loci in a meiotically heritable fashion. If allelic, such non-Mendelian interactions are referred to as paramutation. In the non-allelic case, the term 'heritable trans-silencing' may be used. Paramutation has been studied extensively for four maize loci that encode transcriptional regulators of pigment biosynthesis [1]. Paramutation has also been investigated in the Arabidopsis *PAI* gene family, at the *a1* and chalcone synthase (CHS) transgene loci in petunia, and in a number of other cases [2–4]. The Arabidopsis resistance gene *BAL/CPR1* displays a related form of epigenetic instability [5,6]. Interactions resembling paramutation also occur among non-allelic transgene loci with DNA sequence homology. In these cases one master locus tends to suppress the expression of its target locus ('trans-silencing') [7–11]. Paramutation and trans-silencing are related processes. For example, an inverted repeat allele of the tryptophan biosynthetic gene *PAI* resident in the WS ecotype of Arabidopsis silences homologous *PAI* alleles, as well as unlinked *PAI* genes, from the Columbia ecotype [2]. Likewise, complex synthetic transgenes composed of *PAI* inverted repeats were able to trans-methylate homologous, yet non-allelic, target loci [12]. Similar events have been observed in other species [e.g. [13]].

Epigenetic activity, defined here as partnership in heritable trans-silencing or paramutation, is not predictable from the DNA sequence alone, and its molecular basis is incompletely understood. Arguably the most widely implicated factor is the presence of complex DNA sequence repeats. These repeats are often part of the trans-silencer locus or paramutagenic locus or its target [10,14–17]. However, the repeat can sometimes be located at a considerable distance from the affected locus itself, as has recently been shown for the single-copy maize *b1* gene [18]. Other than transcriptional gene silencing, paramutation is only weakly correlated with DNA methylation. The methylation status of the maize *r1* genes and Arabidopsis *PAI* genes is altered upon paramutation [12,19], while that of maize *b1* is not [1], except, in the non-conventional way, in the distal paramutation-control region of *b1* [18]. Conversely, extensive exposure of a wild-type Arabidopsis *SUPERMAN* allele to a heavily methylated and transcriptionally silenced epiallele did not reveal any trans-silencing [20].

The stochastic nature of gene silencing has been documented on numerous occasions, e.g. [9,21], including in one of the initial descriptions of cosuppression [22]. However, with specific regards to epigenetically active transgene loci, it is often not transparent whether their silencing behavior was stable over successive generations, nor when and how individual loci acquired their epigenetic activity. Certain loci are known to acquire their epigenetic activity spontaneously [4], suggesting an epigenetic control mechanism. For instance, at the *b1* locus, one specific allele can switch spontaneously from a non-paramutagenic, transcriptionally active, state to a paramutagenic, transcriptionally silenced, state [23]. Whether the same is true for trans-silencing is not well established.

Whether the imprint imposed by a paramutagenic locus is relayed effectively from the first target locus to a secondary target locus is a variable characteristic of paramutation systems, and this has implications for the speed of epiallele conversion within outcrossing populations. Paramutable maize alleles of *b1* and *pl1* are highly effective in relaying such an imprint [24,25] (also see [3]), whereas other loci, although sensitive to imprinting, are less effective [26] or apparently ineffective [8] in relaying the imprint to secondary targets. Likewise, Arabidopsis *PAI2* and *PAI3* genes that have been trans-methylated by the *PAI1*/*PAI4* locus do not transfer their methylation status to naive singlet genes [12]. In fact, there are few well-documented cases for the non-allelic relay of trans-silencing ability [11,24]. Does this amount to an operational difference between allelic (paramutation) and non-allelic (trans-silencing) interactions, or does it simply reflect a lack of data?

A translational fusion between green fluorescent protein (GFP) and the Arabidopsis Constitutive Photomorphogenesis1 protein (COP1) has been established as a reporter for trans-silencing events in Arabidopsis [27]. A cauliflower mosaic virus 35S promoter driven *GFP-COP1* locus, named C73, exemplifies an epigenetically active trans-silencer locus. C73 contains at least three copies of a T-DNA comprising the *35S:GFP-COP1* gene as well as a kanamycin resistance gene driven by the nopaline synthase promoter (*nos:nptII*). The C73 locus is silenced transcriptionally (TGS) but retains partial expression of both *nptII* and *GFP-COP1*. *GFP-COP1* remains expressed in the seedling root but is largely silenced in the shoot. Other *35S:GFP-COP1* loci become transcriptionally silenced upon exposure to the C73 locus (Fig. 1). The trans-silencing activity of C73 is conveniently visualized by its negative effect on the cop1-like cosuppression phenotype that is associated with the target locus. For example, L91 is a 'single T-DNA' locus that causes homozygosity-dependent cosuppression of its *GFP-COP1* transgene and of the endogenous *COP1* gene at the rosette stage of development (type L=late silencing), which in turn results in a characteristic dwarfing and other aspects of a *cop1*-like phenotype [28]. In contrast, E82 (type E=early) is an oligomeric T-DNA locus that causes homozygosity-dependent *COP1* endogene silencing at the seedling stage, in particular deetiolation in dark-grown seedlings. E82 also causes essentially dominant cosuppression at the adult stage. Both L91 and E82 are silenced posttranscriptionally (PTGS) and both are representative for a larger group of loci with similar transgene silencing characteristics. Note that PTGS is often homozygosity-dependent [29]. The trans-silencing of the PTGS loci L91 or E82 by the C73 silencer locus is most easily apparent by the loss of the typical *cop1*-like cosuppressionphenotype (Fig. 1) [27]. Similar results were obtained for another trans-silencer locus, C97.

The experimental strategy of monitoring heritable epialleles via their effect on visible cosuppression patterns has been pioneered in the chalcone synthase (CHS) family of petunia and Arabidopsis [4,10,30]. For comparison, *CHS* trans-silencing offers exquisite sensitivity, in part due to the cell autonomy of its pigmentation phenotype, whereas *COP1* essentially yields a whole-plant phenotype. Petunia *CHS* also lends itself to detect somatic transitions in epialleles due to its indeterminate growth habit, whereas Arabidopsis *COP1* is more suitable for monitoring early epigenetic activity in seedlings and rosette plants.

We have begun to define the determinants of epigenetic activity of the *GFP-COP1* loci. The trans-silencers as well as their targets reside in single-copy, gene-rich regions that are only sparsely populated with repetitive or transposable elements, features that have been shown to mediate

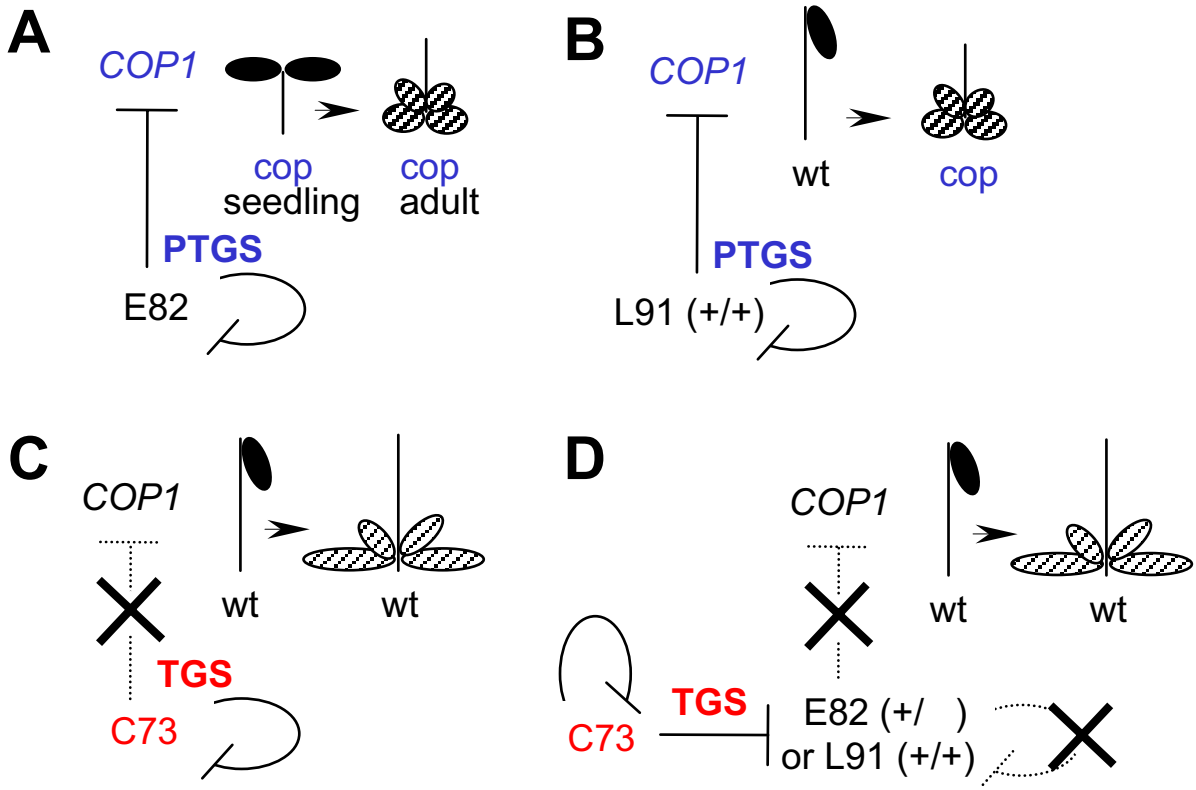

Figure 1
Principle of interactions between *GFP-COP1* transgenes and the *COP1* endogene [27]. The symbols next to the *COP1* endogene represent dark-grown seedling and light-grown adult phenotypes. **(A)** E82 causes dominant transgene silencing and *COP1* endogene cosuppression by PTGS. Note however that the cop1-like seedling phenotype is restricted to homozygous plants. **(B)** L91 causes homozygosity dependent transgene silencing and endogene cosuppression by PTGS. **(C)** C73 plants of the T3 generation and beyond are transcriptionally silenced and do not display endogene cosuppression. **(D)** C73 trans-silences E82 and L91 by TGS and thus suppresses PTGS by the E82 and L91 target loci.

epigenetic activity in other cases [18,31,32]. Therefore trans-silencing of *GFP-COP1* is probably not mediated by transgene flanking sequences [27]. In contrast, as with other genes cited above, trans-silencing ability is correlated with transgene locus structure, given that the C73 and C97 trans-silencers contain multiple T-DNAs while their targets, E82 and L91, are essentially dimeric and monomeric, respectively.

Here we report that the Arabidopsis C73 and C97 trans-silencer loci displayed TGS and trans-silencing after first passing through a transitory phase of PTGS over the course of the first two transgenic generations. A similar epigenetic instability of the initial PTGS phenotype was typical for a subset of other oligomeric loci, but was never observed with monomeric *35S:GFP-COP1* loci. It is this instability of cosuppression that originally prompted us to categorize certain loci as type C rather than type E. We also characterized the heritability of the imprint imposed

by the C73 trans-silencer locus with respect to (a) cosuppression of the *COP1* endogene and (b) a characteristic yet unusual expansion of transgene expression at the target locus. Finally, our data suggest that the L91 target locus acquires limited trans-silencing activity of its own after exposure to C73, which is an unusual case for transgenes. However, these data must be interpreted in light of another novel observation, namely that even relatively simple, transcriptionally active, transgene loci can interact with nonlinear gene dosage effects that have characteristics of epigenetic trans-silencing.

Results

The silencing phenotypes of C73 and C97 were acquired epigenetically

Many of the most potent epigenetically active loci are endogenous rather than transgenic and, therefore, their early epigenetic history is not completely known. In contrast, transgenes have a defined date of origin within a plant lineage. In our transgene-based system, the silencer loci *GFP-COP1* C73 and C97 underwent an apparently spontaneous transition in their silencing behavior between the T2 and T3 generations (Fig. 2A). In detail, the progeny of the primary transgenic plant, T1 plant C73, which possessed a single transgene locus as determined by segregation of antibiotic resistance, segregated almost one quarter cop1-like seedlings in the dark-grown T2 generation. This dosage dependence of endogene silencing was the same as for the PTGS locus E82 (Fig. 1). Unambiguous evidence for a cosuppression of transgene and endogene, as opposed to a dominant negative effect of the transgenic protein, has previously been shown for line L4 and others [27], and COP1 endogene suppression was also associated with GFP-COP1 silencing in T2 generation type C lines (not shown). At the adult stage, 12% of T2 plants in line C73 were visibly cosuppressed. In contrast, the T3 generation, derived by selfing of T2 plants and known by run-on transcription assay to be silenced by TGS [27], had few cop1-like seedlings and no cop1-like adult plants. Similar results were obtained for line C97. The C97 line is the only line in this work that contains two unlinked loci [27], which may have contributed to the high incidence of PTGS in T2 seedlings. Neither of these loci retained its endogene silencing ability in the T3 (Fig. 2). No *COP1* cosuppression was observed beyond the T3 generation of C73 or C97 (not shown). Two additional oligomeric *GFP-COP1* loci, C11 and C71, which have not been tested for their trans-silencing behavior, also changed from a cosuppressing state in the T2 generation to a non-cosuppressing state in the T3 (Fig. 2A). In contrast, none of four (single T-DNA) type L loci lost their endogene silencing ability (Fig. 2B). The apparent increase in adult COP1 cosuppression from the T2 to the T3 of some type L lines was not statistically significant. The type E loci E82 and E83 were also stable (Fig. 2C). Together, these results suggest that PTGS in certain oligomeric T-DNA loci is unstable and has a tendency to progress to TGS. In line C73, the transition probably began in T2 seedlings, as suggested by the small fraction of adult T2 plants with a cop1-like PTGS phenotype.

Transgene locus structure of the E82 locus

In order to distinguish the L91 and E82 loci more confidently in subsequent experiments, we defined the structure of the E82 locus in more detail. The L91 locus is a simple T-DNA locus at a defined chromosomal location. In contrast, Southern blots probed with the T-DNA right border (RB) revealed two fragments for E82 [27]. Early attempts to isolate the E82 flanking sequence by TAIL-PCR repeatedly recovered a fortuitous junction between a left border (LB) and a partial T-DNA truncated near the 3' end of the *GFP-COP1* gene. A schematic drawing of the E82 locus structure incorporating all of these data as well as data from blots probed with LB and *COP1* sequences (not shown) is presented in Fig. 3. This model was subsequently confirmed in two ways: an *NdeI* restriction site predicted from Southern blots was confirmed in the flanking sequence [27]; and PCR amplification with specific primers across the flanks of the transgene and across the internal LB-RB junction resulted in the expected product lengths (Fig. 3). Thus, we propose that the E82 locus consists of a direct repeat, which is joined at the tail end by a partial T-DNA in an inverted orientation.

Trans-silencing by C73 results in variable novel transgene expression patterns, including spatial expansion of transgene expression

The C73 trans-silencer locus caused transcriptional silencing of two target loci tested, L91 and E82, which is most easily detected by a suppression of the typical endogene cosuppression of the target loci (Fig. 1) [27]. As expected, the spatial expression pattern of L91's *GFP-COP1* gene was restricted, though not completely abolished, upon exposure to C73 (not shown, see Fig. 5D below). In contrast, F2 families segregating for C73 and E82 contained many plants with an expanded spatial pattern of expression, namely 'reactivation' of transgene expression in the cotyledons and the first two pairs of leaves, especially in trichomes (Fig. 4 panels B, E, H, and K). For comparison, the naive E82 locus as well as C73 alone were silenced in the leaves (Fig. 4A,4D,4G and 4J and Fig. 4C,4F,4I and 4M). Such a reactivation had previously been observed in the dihybrid C73/E82 F1 parent plants [27]. The reactivated expression was subsequently ascribed to the E82 locus, rather than C73, by testing F2 segregants lacking C73 (see Figure 5 below). In summary, silencing of L91 and E82 by C73 was incomplete, as especially E82 recovered partial transgene expression in organs where it was not normally expressed.

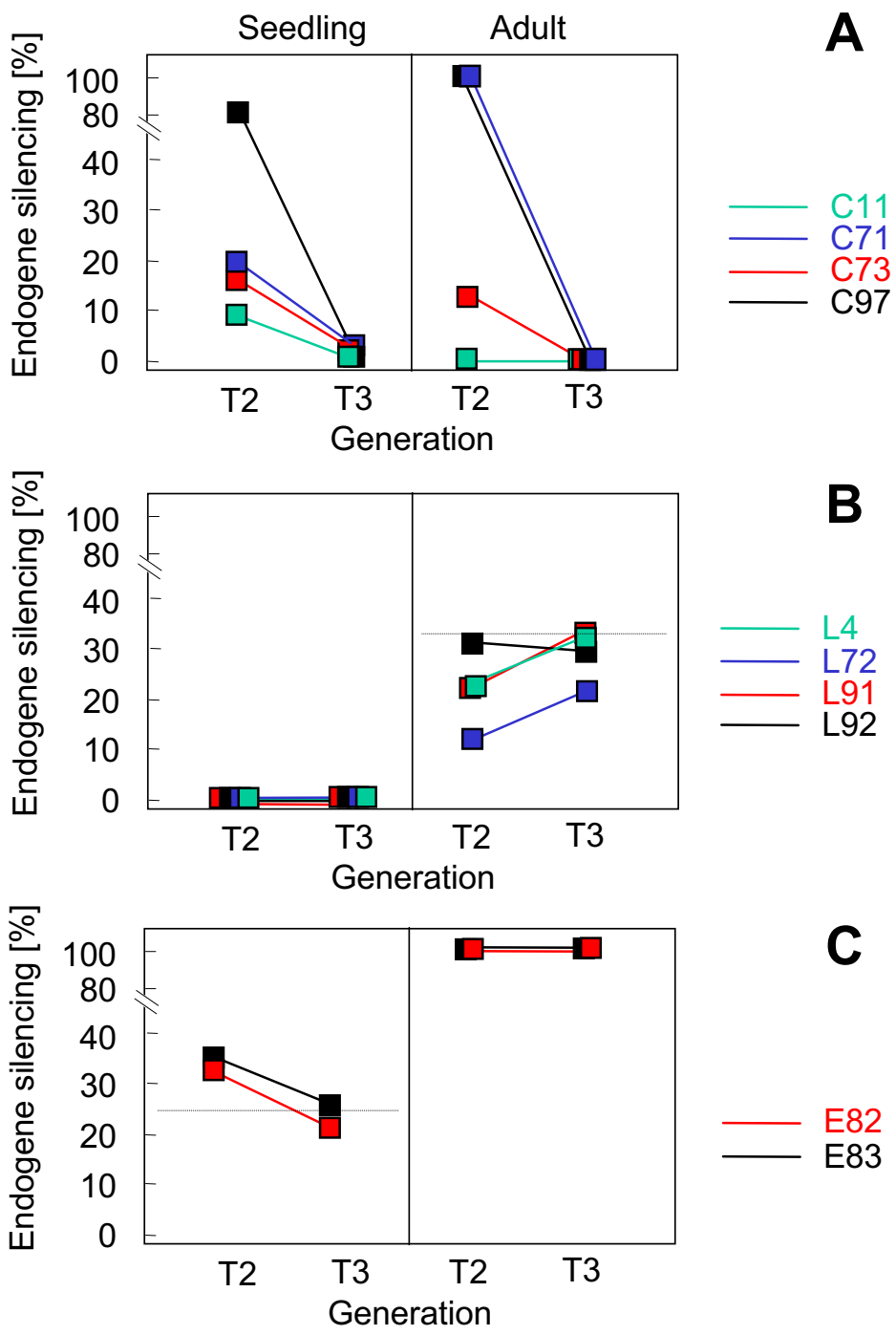

Figure 2
Spontaneous epigenetic transition between two silencing states. The percentage of cop1-like endogene silenced plants is given for seedling and adult stages of the T2 and T3 generations of **(A)** type C loci, **(B)** type L loci, and **(C)** type E loci. Note: Adult plants had been preselected on kanamycin. Thus, homozygosity dependent silencing (typical for type L adults, see horizontal line) gives 33% silenced plants. Seedlings were scored independent of kanamycin selection. Hence, homozygosity dependent silencing (typical for type E seedlings) gives 25% silenced plants.

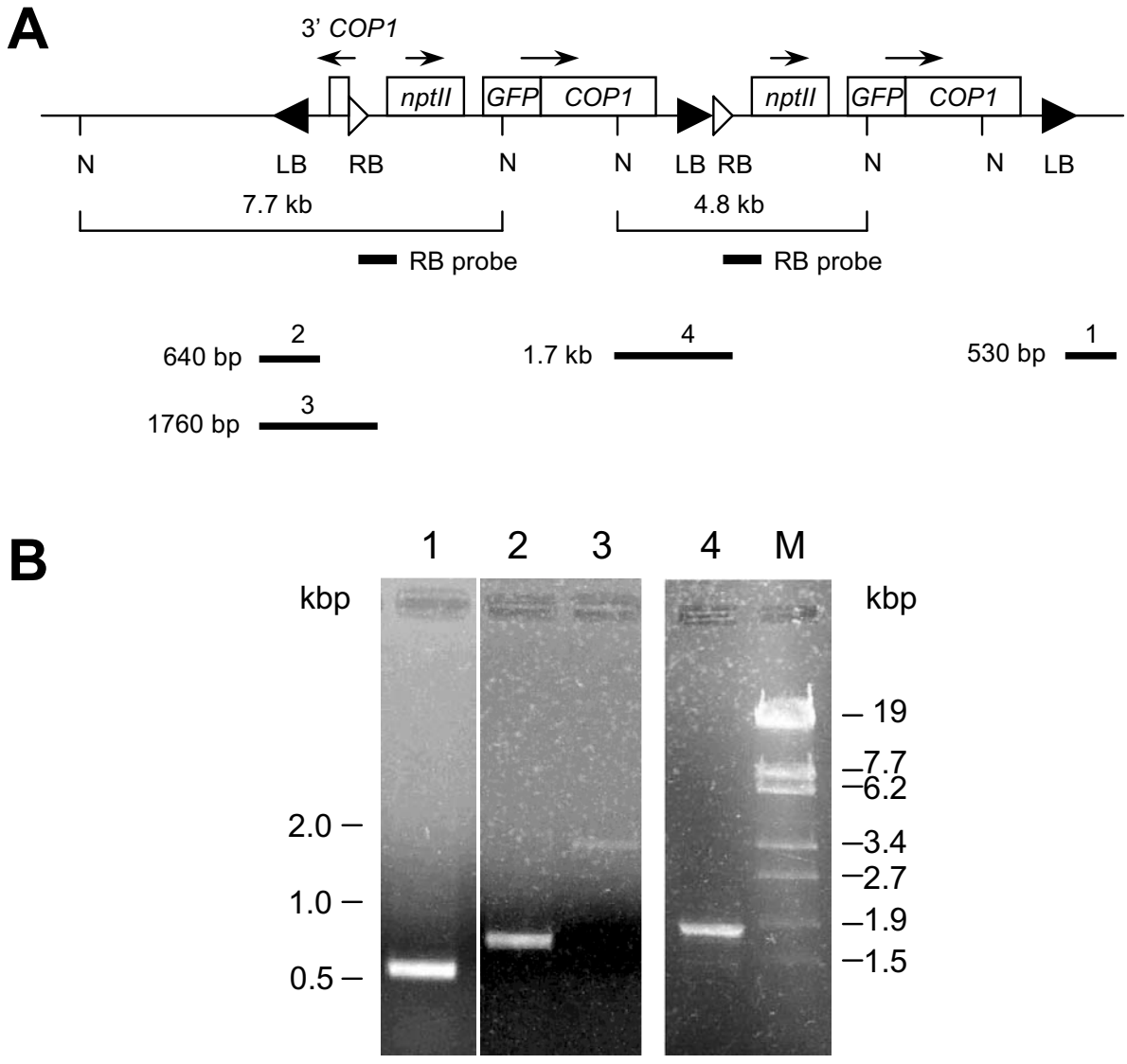

Figure 3

Structure of the *GFP-COP1* locus E82. (A) The arrangement shown at the top was suggested by Southern blot analysis of NdeI (N) digested genomic DNA with a right border probe as indicated. In addition, PCR assays using sequence specific primers resulted in the predicted products as indicated by solid lines. Sizes of predicted PCR products are given in basepairs. Digits above the PCR products refer to the lane number in panel (B) showing the relevant fragment. The E82 locus lies on Chromosome 1 at basepair 42,325 of BAC T6A9 (Genbank: AC064879). **(B)** Ethidium bromide stained gel images showing predicted PCR products generated from the border sequences (lanes 1 to 3, fragment sizes indicated at left) and the internal RB/LB junction (lane 4, see marker at right).

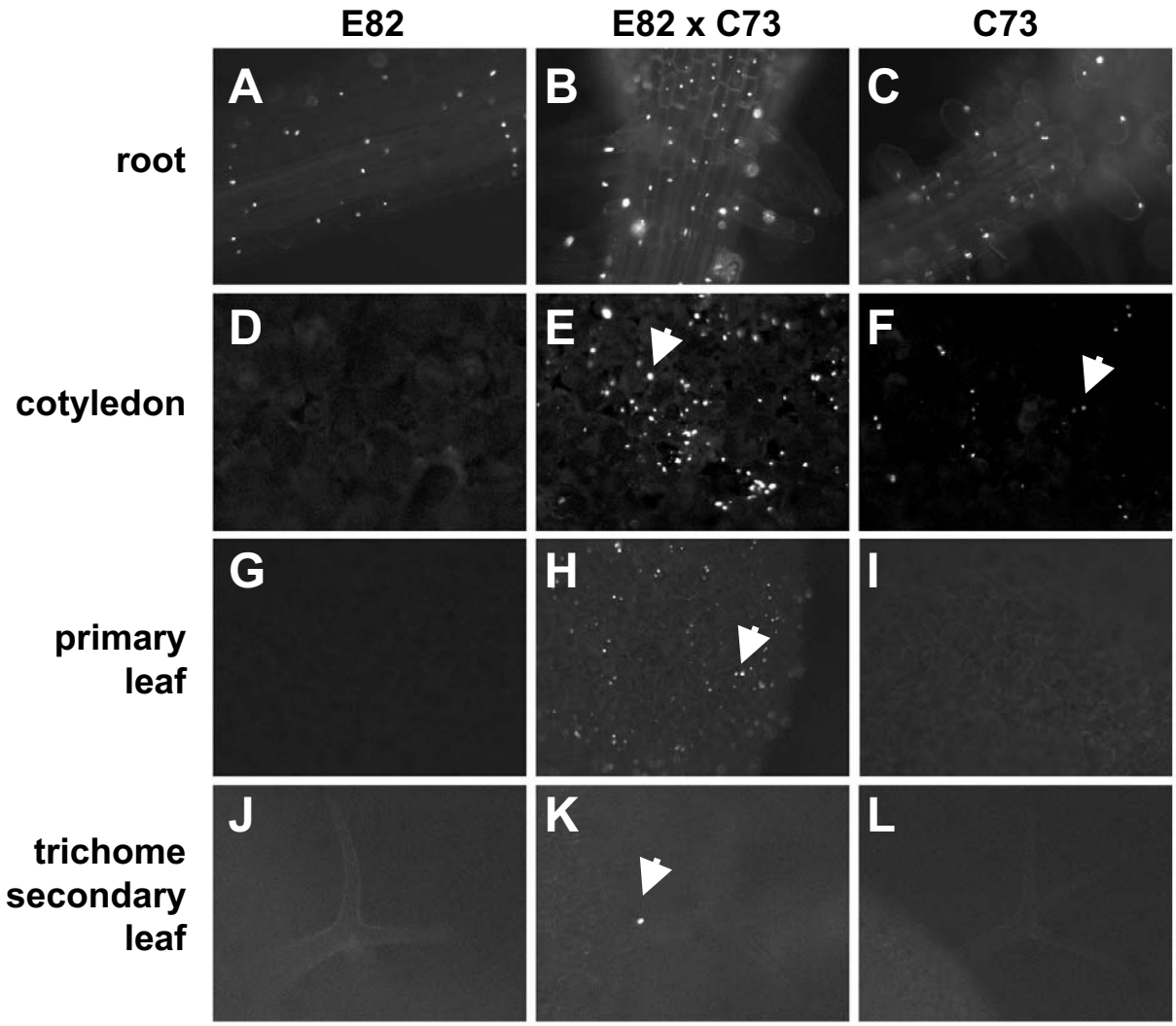

Figure 4
Reactivation of transgene expression from the post-transcriptionally silenced E82 locus after exposure to *GFP-COP1* C73. Epifluorescence micrographs of whole-mounted seedlings are shown. Note that GFP-COP1 protein accumulates in the form of a single inclusion body per cell. Left column (panels **A, D, G,** and **J**): Control: Progeny of a *GFP-COP1* E82 hemizygous plant. GFP-COP1 was active only in the root. Central column (panels **B, E, H,** and **K**): Progeny of a *GFP-COP1* C73 × E82 di-hybrid plant. GFP-COP1 was often active in the root, cotyledon, and first and second sets of leaves. Arrows highlight the reactivated *GFP-COP1* in the cotyledon, leaf epidermis, as well as in trichomes of the second set of leaves. Right column (panels **C, F, I,** and **L**): Control: Progeny of a *GFP-COP1* C73 hemizygous plant. GFP-COP1 was active in the root and in guard cells (note paired dots) of the cotyledon.

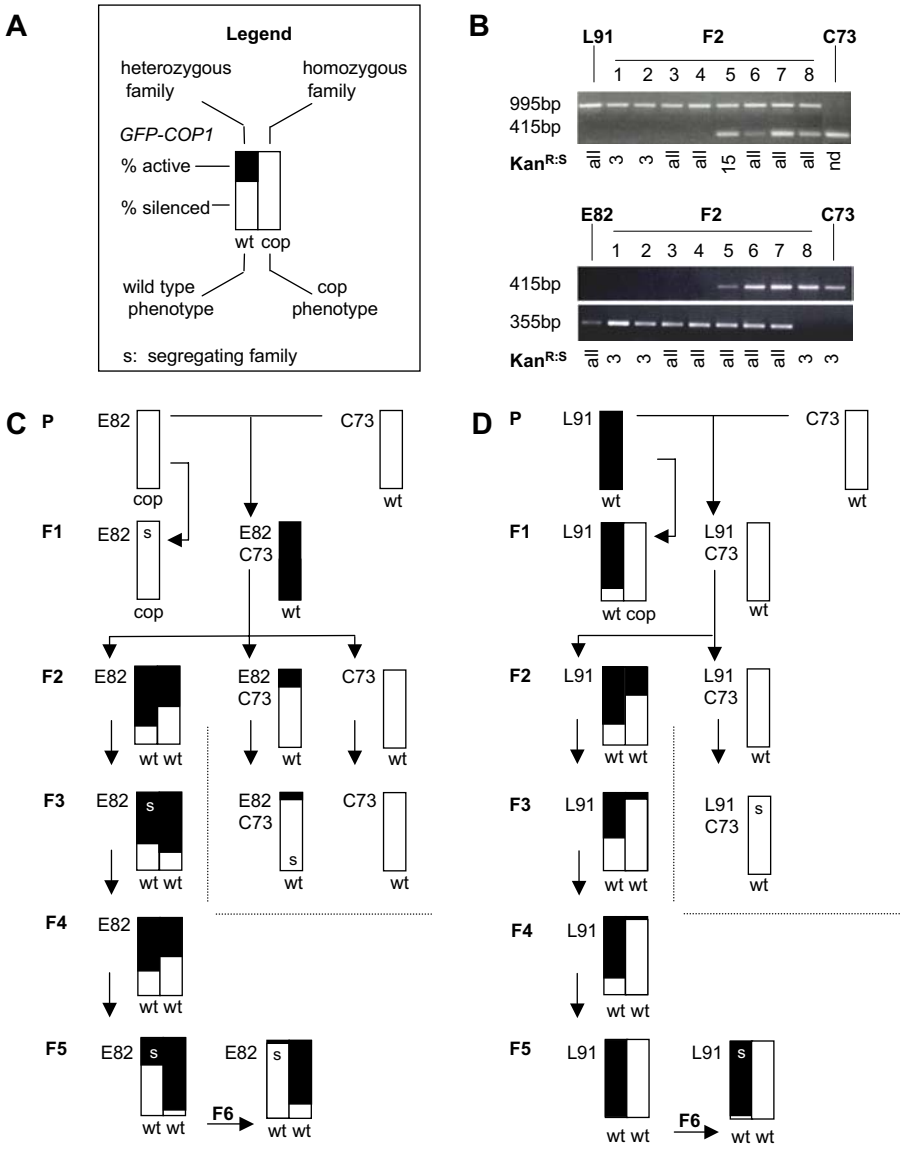

Figure 5

The trans-silencing of L91 and E82 by C73 is stably inherited for up to five generations. Trans-silencing suppresses the *cop1*-like PTGS phenotype of L91 homozygotes and of E82 plants and causes reactivation of E82 *GFP-COP1* expression. **(A)** Legend to illustrate the phenotypic scoring scheme. **(B)** Diagnostic PCR assay. DNA from individual F2 plants was subjected to PCR with primers specific for L91 and C73, or E82 and C73, as indicated. Lane 1: L91 or E82 control. Lane 10: C73 control. Lanes 2 to 9: Eight individual F2 plants. The gene dosage for L91 and E82 was defined by the segregation ratio of the kanamycin resistance marker in the F3 progeny ($Kan^{R:S}$, either 3:1, 15:1, or all). **(C, D)** Transgene expression (bars) and endogene silencing phenotypes (cop, wt) of parental controls, dihybrid F1 plants or their single-transgene control siblings, individually genotyped F2s, as well as their selfed progeny. **(C)** E82 and C73 and **(D)** L91 and C73. Shading of the bars reflects the percentage of plants with active or silenced *GFP-COP1* transgene expression. In the case of heterozygous lineages (s) the average family size was 32. Note: In the F4 generation a small fraction of plants with a cop1-like cosuppression phenotype was seen among the majority of wild-type plants.

These results underscore the similarity between trans-silencing and paramutation, because in both cases epialleles are often silenced incompletely and may also adopt novel regulatory patterns [4,10], for example light inducibility [33]. Perhaps, silencing at the dimeric E82 locus involves a hierarchical relationship between its two intact T-DNAs. The C73 locus may preferentially target a postulated 'master' T-DNA, which in turn loses its ability to post-transcriptionally silence the 'subordinate' T-DNA within the E82 locus.

Two target loci of C73 maintain their epigenetic imprint heritably for up to five generations

A diagnostic PCR assay was developed for the L91, E82, and C73 loci to unambiguously identify F2 segregants containing a trans-silenced L91 or E82 target locus but lacking the C73 silencer locus (Fig. 5B; compare lanes 1–4 with lanes 5–8). In addition, hemizygous segregants were distinguished from homozygotes by segregation of the kanamycin resistance gene in their selfed progeny (compare lanes 1–2 with 3–4). Subsequently, the rate of phenotypic reversion from trans-silencing was observed for hemizygous and homozygous lineages of E82 and L91. A legend for scoring the *cop1*-like cosuppression and *GFP-COP1* transgene silencing patterns is shown in Fig. 5A. Unless exposed to C73, E82 plants are always *cop1*-like and silenced for GFP-COP1, and the same is true for homozygous L91 plants. In contrast, E82 plants and homozygous L91 plants were wild type-like, rather than cosuppressed, in the F2 if the transgene had been exposed to C73 in the F1 (Fig. 5C and 5D, previously summarized in [27]). Both the reactivation of E82's GFP-COP1 transgene expression as well as suppression of endogene silencing were maintained for up to four additional generations in the absence of C73 in hemizygous and homozygous families, while C73 remained silenced (Fig. 5C). The L91 lineage also maintained its imprint from C73 over up to five generations. Specifically, the cop1-like cosuppression phenotype typical for homozygous L91 plants remained suppressed (Fig. 5D).

Although the imprints on the E82 and L91 loci proved to be fairly stable, reversion was observed in two ways. First, a small fraction (less than 20% expected for full reversion) of cop1-like E82 plants and L91 plants appeared in the F4 generation (not reflected in Fig. 5); and second, the GFP-COP1 expression of E82 gradually reverted back from the reactivated pattern to the silenced pattern especially in hemizygous families (Fig. 5C). The transgene expression data for the imprinted L91 lineage were less informative. However, it appears that the hemizygous L91 lineage gradually recovered its *GFP-COP1* expression but without reaching the threshold required for posttranscriptional cosuppression of the *COP1* gene.

Imprinting relay: Do trans-silenced E82 and L91 loci acquire trans-silencing ability?

Our data suggested that the C73 locus may have acquired its trans-silencing activity as part of an epigenetic transition from type E to type C silencing between the T1 and T3 generations. Thus, it is reasonable to ask whether the trans-silencing activity be farther transferable from the type C locus to other loci. It seemed problematic to test whether the trans-silenced L91 locus (henceforth termed L91') can paramutate a naive L91 allele, because we cannot distinguish between the L91' master and the L91 target at the molecular level. Instead, L91' was tested for its effect on a naive E82 locus and E82' was tested against L91, because these loci are distinguishable by diagnostic PCR assays. Because L91' and E82' no longer cause *COP1* cosuppression, it was easy to monitor the interaction by following the cosuppression phenotypes of the naive E82 and L91 loci. Figure 6 provides an overview over the crossing scheme, and experimental data are presented in Tables 1 to 3. Significantly, the L91' locus suppressed the *COP1* endogene silencing by the E82 target locus in the majority of F1 dihybrid plants, whereas a naive L91 locus did not (Table 1). Although only four relevant plants were recovered by genotyping in the L91 × E82 control experiment, the entire L91 × E82 family contained a large fraction of silenced plants, larger than with L91' and E82, which was compatible with an additive interaction between L91 and E82 (not shown). As expected, at the level of transgene expression, both L91'/-; E82/- and L91/-; E82/- plants were silenced (Table 1; Fig. 6). Likewise, in the F2 progeny, L91' continued to suppress the endogene silencing by E82 (Table 2; Fig. 6), which is normally expected to affect about three quarters of F2 progeny. Importantly, upon genotyping of individual F2 plants, five wild type-like plants were identified that carried only E82 and no L91' locus, confirming that L91' can leave a heritable imprint on E82 (Table 3). However, two additional pieces of data distinguish the trans-silencing activity of L91' from that of C73. First, the F2 families clearly contained plants displaying endogene silencing. In the one case tested, this was due, not surprisingly, to an E82 locus that had escaped from L91' and resumed its endogene silencing (Table 3). Therefore, L91' is a less effective trans-silencer than C73 [27]. Second, and more surprisingly, even naive L91 and E82 loci, which had shown no trans-silencing in the F1, displayed a loss of the E82 cosuppression phenotype in the F2 (Table 2). Instead of an expected Mendelian ratio of 87% *cop1*-like plants, only 38% were observed, a significant shortfall. Genotyping of individual F2 plants revealed a stochastic escape from *COP1* endogene silencing in plants containing both E82 and L91 and including even a single E82 plant that lacked L91 (Table 3). Such a loss of cosuppression has not been observed among hundreds of plants containing just E82 alone (our unpublished data). These

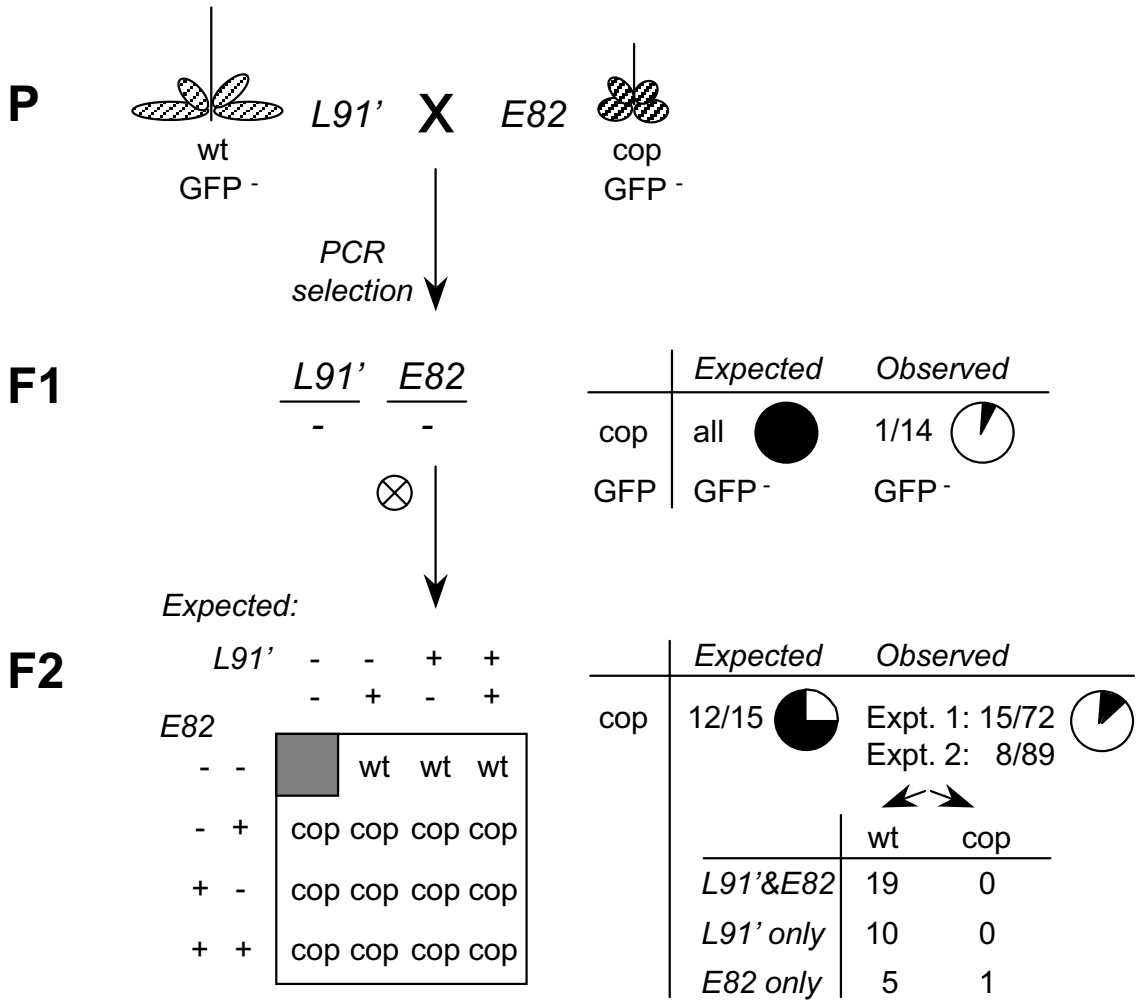

Figure 6
Test for relay of trans-silencing ability from C73 via L91' to E82. For details see text and Tables. Under 'Expected' the figure shows the predicted results under the null-hypothesis that L91' would *not* trans-silence E82. This is referred to as an additive interaction.

data suggest an epigenetic modification of E82 after exposure to a naive L91 locus.

In contrast to the epigenetic activity of trans-silenced L91', E82' had no such activity. When E82' was combined with L91 both loci cooperated to cause endogene silencing, as seen with unmodified E82 and L91 (Table 1). This was unexpected given that E82' caused little or no endogene cosuppression with L91' or on its own. Cooperative cosuppression between bona fide imprinted E82' and a naive L91 locus was again detected in the F2 progeny (Table 2). Thus, E82' was clearly unable to trans-silence a naive L91 locus and in fact seemed to lose its imprint when exposed to L91; therefore these families were not analyzed further. E82' also did not trans-silence a naive E83 locus (not shown).

Taken together, these data allow three conclusions. First, experiments with naive L91 and E82 demonstrated that even relatively simple transgene loci associated with PTGS can display non-additive gene dosage effects, as apparent by heritable suppression of PTGS. Second, the imprint left

Table 1: Exposure to C73 differentially modifies the trans-silencing activity of the L91 and E82 loci – Gene expression data from individually genotyped F1 hybrid plants. See Fig. 6 for details.

F1 genotype	Endogene[1]			*GFP-COP1* transgene [% of plants]			
	Expected	Observed		Observed			
	cop	cop	wt	Root	Cotyledon	1°Leaf	n[2]
L91'/-; E82 /-	all	1	13	100	0	0	11
E82'/-; L91 /-	none	16	1	100	80	47	15
Controls:							
L91 /-; E82 /-	all	4	0	100	0	0	3
L91'/-; E82'/-	none	0	26	100	88	75	16

[1] The *COP1* endogene phenotype is scored in adult plants. [2] n: number of plants

Table 2: The PTGS-mediated endogene cosuppression phenotype of the E82 locus is reduced by trans-silenced L91' as well as by naïve L91.

Grandparents	F2 family endogene silencing phenotype				
	Endogene		[%] cop		Remarks[1]
	cop	wt	Observed	Expected	Excess of
L91' × *E82*	23	138	14%	75%	wt plants
E82' × *L91*	30	42	42%	27%	cop plants
L91 × *E82*	38	73	38%	87%	wt plants
L91' × *E82'*	1	17	6%	0%	one cop plant

[1] Data were Chi-square tested under the null-hypothesis that L91' and E82' do not trans-silence their target loci, i.e., an additive interaction between the partner loci. Thus, in the case of L91 × E82, the only genotype escaping cosuppression should be L91 /-; E82 (-/-), which occurs at a frequency of 2/15 (13%). Note that entirely non-transgenic plants are not considered because of counterselection. Imprinted loci are not expected to cause endogene silencing (see Fig. 5). Thus, 12/15 of L91'/E82 family members, 4/15 of E82'/L91 family members and none of L91'/E82' family members are expected to be cop1-like. An excess of wild-type plants is inconsistent with an additive interaction and thus suggests trans-silencing activity by the L91' or L91 locus.

Table 3: Both L91' and L91 can heritably suppress the cop-like endogene silencing by the E82 locus.

F2 family	Individual plants			
	Phenotype	Transgene locus present		
	adult	L91 & E82	L91 only	E82 only
L91' × *E82*	wt: 34	19	10	5
	cop: 1	0	0	1
L91 × *E82*	wt: 9	6	2	1
	cop: 8	3	3	2

Individual F2 plants from Table 2 were tested for transgene loci by diagnostic PCR assays.

on the L91 locus by the C73 trans-silencer did modify L91's epistatic interaction with a non-allelic target locus, E82, as seen in the F1 generation. However, it remains to be determined whether this trans-silencing effect is more likely to be heritable when L91 is in an imprinted state (L91') than in the naive state (L91). Third, the imprint on the E82 locus (E82') was more labile than the imprint on L91' and showed no evidence of being transferable.

Discussion

Epigenetic imprints affecting the expression of nuclear genes differ in the efficiency with which they are relayed onto homologous sequences. In the classical case of paramutation at the maize *b1* locus, the imprint is relayed with 100% efficiency [24]. That is, a paramutated allele is turned into a paramutagenic allele. In contrast, to our knowledge, no trans-silenced transgene has been shown to acquire heritable trans-silencing ability suggesting that perhaps trans-silencing ability is more difficult to relay between non-allelic transgenes than between allelic loci. However, the endogenous *p1-rr* allele of maize did acquire paramutagenicity after exposure to a specific *P1*-enhancer transgene [11]. Against this background we investigated the possible relay of an imprint triggered by the Arabidopsis C73 trans-silencer locus, asking specifically whether targets trans-silenced by the C73 locus (a) acquired trans-silencing activity, and (b) were able to pass their imprint on to a naive target locus in a heritable fashion.

In summary, the imprinted E82 locus (E82') displayed no significant ability to trans-silence a naive L91 locus. In contrast, L91' was initially able to trans-silence E82, as judged by suppression of the endogene cosuppression phenotype associated with naive E82; importantly, the unmodified L91 control locus did not have this ability (Table 1). Thus, we demonstrated aspect (a) of the imprinting relay. Moreover, the suppression of silencing at the E82 locus was partially but not fully heritable given that some but not all E82 segregants lacking L91' were wild-type (Table 3). Yet, these data fall short of proving aspect (b) of the imprinting relay because, surprisingly, the combination of naive L91 and E82 loci also caused non-linear gene dosage effects that could result in the heritable trans-silencing of the E82 locus in at least one instance. Whether L91' might paramutate an allelic L91 locus remains to be tested. The non-linear gene dosage effects between L91 and E82 were surprising to us because in tobacco, similar experiments conducted with *35S:GUS* transgenes did not raise the suspicion of epigenetically heritable effects [34].

It is informative to compare the epigenetic interactions of the *35S:GFP-COP1* transgenes with those of the *PAI* (trans)genes, the only other well-characterized system in Arabidopsis [12]. Both gene sets consist of relatively simple repeat structures and singlet loci, which reside in essentially single-copy environments of Arabidopsis ([27] and unpublished observations). The first comparison relates to the speed of epigenetic change. Here, combining a master locus (C73) with a non-allelic singlet locus possessing 100% sequence identity (L91) resulted in immediate trans-silencing, as seen by a reduction of GFP-COP1 expression and block of cosuppression, which was stably maintained in the presence or absence of the master locus. In contrast, in the *PAI* gene family, combining the master locus (WS ecotype *PAI1*/*PAI4*) with a non-allelic singlet target locus possessing 100% sequence identity (Columbia *PAI2*) resulted in methylation after two generations of heterozygous contact, and methylation became more pronounced after another two generations [12]. Likewise, in the petunia *CHS* gene family, interaction between an epiallele of the direct-repeat locus, CHS41, with two types of target loci, either an unlinked inverted repeat locus or a more naïve allele of CHS41, was initially additive and only became suppressive in the second generation [4,10].

The second comparison relates to the relay of epigenetic activity. In our system, a trans-silenced singlet gene (L91') was able to trans-silence a naive homolog (E82) within a single generation. In contrast, a trans-methylated singlet *PAI2* gene did not trans-methylate a naive allelic *PAI2* gene within three generations [12]. Additional experimentation may eventually provide an answer to the question of what controls the efficiency of such epigenetic interactions.

Cosuppression of the endogenous COP1 gene is a consequence of posttranscriptional silencing, while transcriptionally trans-silenced loci do not cosuppress COP1 [27]. In our hands, a subset of four *35S:GFP-COP1* transgene loci shifted from a cosuppressing state, bona fide PTGS, to a non-cosuppressing state, i.e. TGS, within two transgenic generations. These loci are therefore referred to as 'complex' loci. Note that type L and type E loci did not lose their PTGS phenotype (Fig. 2B). This includes the L72 locus, which has a reduced penetrance of cosuppression that may be attributable to its pericentromeric location [27]. Intriguingly, both trans-silencer loci, C73 and C97, belong to the 'complex' group, adding weight to the proposition that, like paramutagenicity, trans-silencing activity is encoded epigenetically. Vice versa, this transition raises the question whether the originally strong transcription of the *GFP-COP1* genes, or perhaps the ensuing PTGS phase, somehow sets the stage for the subsequent trans-silencing activity, especially if multimeric T-DNA loci are involved. PTGS and trans-silencing are similarly intertwined in other cases, for example in the epimutable petunia CHS41 locus [10]. PTGS often leads to DNA methylation, although primarily in coding regions [35]. However, DNA methyl-

ation of promoters, more easily aligned with transcriptional silencing, can be RNA mediated if suitable double stranded RNA versions of the promoter sequence are transcribed, either fortuitously or by design [36–40]. Therefore, there may be a natural tendency for PTGS loci to mature to TGS and associated trans-silencing, which in turn could prove problematic in applying PTGS in commercial plant breeding programs. Our data are certainly consistent with this notion. Such hypotheses are testable with the model system we have established.

Conclusions

1. Posttranscriptional gene silencing by structurally complex transgenes loci may be unstable and may be supplanted by transcriptional gene silencing over the course of a few plant generations.

2. Heritable modifications of transgene expression caused by exposure to a trans-silencer locus may be stable for five generations or more.

3. Upon exposure to a trans-silencer locus, certain Arabidopsis transgenes display an alteration in their epistatic interaction with other transgenes. These data suggest that the competence for trans-silencing may be transferred between non-allelic transgenes, reminiscent of the allelic transfer of epigenetic activity during paramutation.

4. Cosuppression phenotypes can serve as sensitive indicators of epigenetic interactions between transgenes in Arabidopsis thaliana.

Methods

Transgenic Arabidopsis lines and plant growth

The GFP-COP1 expression cassette contains a double 35S enhancer, translational enhancer, and 35S terminator. Most GFP-COP1 lines and the GUS-COP1 line L4 have been described [27,41,42]. Unsilenced GUS-COP1 and GFP-COP1 transgenes complement the cop1 mutation [42,43]. The C11 and C71 loci contain at least three and two linked T-DNAs, respectively, as seen by Southern blotting (not shown). Plants were germinated on agar solidified MS medium containing 1% sucrose in either constant light or constant darkness and subsequently grown in soil in growth chambers at 22°C under constant white light from fluorescent tubes.

Test for acquired trans-silencing activity

Trans-silenced L91' and E82' plants that lacked the C73 silencer locus ('prime'-label) were crossed in pairwise combinations with naive E82 and L91 plants. Because trans-silencing of E82 was observed regardless of whether L91' was the male or the female parent, data from reciprocal crosses were pooled. Individual F1 dihybrid plants were inspected for GFP-COP1 and COP1 endogene silencing phenotypes. Representative F1 dihybrids were checked for the presence of two different transgene loci by diagnostic PCR assay and by segregation analysis. F2 segregants containing only the target locus were identified by PCR based genotyping, analogous to the strategy exemplified in Fig. 5. Plants in which GFP-COP1 expression was restricted to roots and cotyledons were regarded as silenced, whereas plants with GFP-COP1 visible beyond the cotyledons were considered active.

PCR assays

The diagnostic PCR assays for the C73 and E82 loci display unique PCR products arising from fortuitous and distinct junctions between the T-DNA right border and the COP1 cDNA, which we recovered in early attempts to isolate flanking sequences. For E82 the primers are ATATTTGCTAGCTGATAGTGACC-3' and GATCCTAGGGGTCTCGTGATTTCTTGTGAT-3'; for C73 they are TGTCAGTTCCAAACGTAAAACGG-3' and GACACATCACAAGATCTTTGTAGTGC-3'. The assay for the L91 locus was based on oligonucleotides specific for the L91 flanking sequence (AGGCACACAAGCCCAAAAAGAC-3') and the RB of the T-DNA [27]. PCR fragments representing the structure of the E82 locus were made as follows: Fragment 1 in Fig. 3B: 5'-AAACAGGATTTTCGCCTGCTGGG-3' (LB) and 5'-ACATCCAAACAGAACGTGCC-3' (Arabidopsis). Fragment 2: 5'-AAACAGGATTTTCGCCTGCTGGG-3' (LB) and 5'-TGCTGTTCAAACCCCAAAATTC-3' (Arabidopsis). Fragment 3: 5'-ATATTTGCTAGCTGATAGTGACC (RB) and 5'-TGCTGTTCAAACCCCAAAATTC-3' (Arabidopsis). Fragment 4: 5'-GGGGGCCATGGAGTATGAAGAGCACGAA-3' (COP1) and 5'-CGGAGAACCTGCGTGCAATCCATC-3' (RB).

Authors' Contributions

HQ designed experiments and collected data underlying Figures 3, 5, and 6 and Tables 1 to 3. AGVA contributed data for Figures 2, 4 and 5 and finalized the manuscript. Both authors read and approved of the manuscript.

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